

**Amendments to the Specification:**

Please replace the paragraph (or section) beginning at page 1, line 4, with the following redlined paragraph (or section):

This application is a CIP ~~of U.S. Application No. 09/684,361, filed October 6, 2000, which is a CIP of U.S. Application No. 09/276,484, filed March 25, 1999, now abandoned,~~ which is a CIP of U.S. Application No. 09/164,223, filed September 30, 1998, now U.S. Patent No. 7,063,854, issued June 20, 2006, all applications incorporated herein by reference in their entirety.

Please replace the paragraph (or section) beginning at page 22, line 12, with the following redlined paragraph (or section):

In one preferred embodiment, the immunological fusion partner is derived from a *Mycobacterium* sp., such as a *Mycobacterium tuberculosis*-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences is described in U.S. Patent Application 60/158,585, now lapsed, the disclosure of which is incorporated herein by reference in its entirety. Briefly, Ra12 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been described (for example, U.S. Patent Application 60/158,585, now lapsed; see also, Skeiky *et al.*, *Infection and Immun.* (1999) 67:3998-4007, incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as soluble polypeptides throughout the purification process. Moreover, Ra12 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One preferred Ra12 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other preferred Ra12 polynucleotides generally comprise at least about 15

consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ra12 polypeptide. Ra12 polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a Ra12 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ra12 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ra12 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ra12 polypeptide or a portion thereof.

Please replace the paragraph beginning at page 33, line 3, with the following redlined paragraph:

T cells may be stimulated with WT1 polypeptide, polynucleotide encoding a WT1 polypeptide and/or an antigen presenting cell (APC) that expresses a WT1 polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the WT1 polypeptide. Preferably, a WT1 polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of antigen-specific T cells. Briefly, T cells, which may be isolated from a patient or a related or unrelated donor by routine techniques (such as by Ficoll<sup>®</sup>/Hypaque-HYPAQUE<sup>®</sup> density gradient centrifugation of peripheral blood lymphocytes), are incubated with WT1 polypeptide. For example, T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with WT1 polypeptide (*e.g.*, 5 to 25 µg/ml) or cells synthesizing a comparable amount of WT1 polypeptide. It may be desirable to incubate a separate aliquot of a T cell sample in the absence of WT1 polypeptide to serve as a control.

Please replace the paragraph beginning at page 39, line 12, with the following redlined paragraph:

Any of a variety of non-specific immune response enhancers, such as adjuvants, may be employed in the vaccines of this invention. Most adjuvants contain a substance designed

to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable non-specific immune response enhancers include alum-based adjuvants (e.g., Alhydrogel, Rehydralgel, aluminum phosphate, Algammulin, aluminum hydroxide); oil based adjuvants (Freund's adjuvant (FA), Specol, RIBI, TiterMax, ~~MONTANIDE®~~ Montanide—ISA50 or Seppic ~~MONTANIDE®~~ MONTANIDE ISA 720; cytokines (e.g., GM-CSF or Flat3-ligand); microspheres; nonionic block copolymer-based adjuvants; dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants AS-1, AS-2 (Smith Kline Beecham); Ribi Adjuvant system based adjuvants; QS21 (Aquila); saponin based adjuvants (crude saponin, the saponin Quil A ); muramyl dipeptide (MDP) based adjuvants such as SAF (Syntex adjuvant in its microfluidized form (SAF-m)); dimethyl-dioctadecyl ammonium bromide (DDA); human complement based adjuvants *m. vaccae* and derivatives; immune stimulating complex (~~iscom~~ISCOM®) based adjuvants; inactivated toxins; and attenuated infectious agents (such as *M. tuberculosis*).

Please replace the paragraph beginning at page 39, line 28, with the following redlined paragraph:

As noted above, within certain embodiments, immune response enhancers are chosen for their ability to preferentially elicit or enhance a T cell response (e.g., CD4<sup>+</sup> and/or CD8<sup>+</sup>) to a WT1 polypeptide. Such immune response enhancers are well known in the art, and include (but are not limited to) ~~MONTANIDE®~~ ISA50, Seppic ~~MONTANIDE®~~ ISA 720, cytokines (e.g., GM-CSF, Flat3-ligand), microspheres, dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants, AS-1 (Smith Kline Beecham), AS-2 (Smith Kline Beecham), Ribi Adjuvant system based adjuvants, QS21 (Aquila), saponin based adjuvants (crude saponin, the saponin Quil A), Syntex adjuvant in its microfluidized form (SAF-m), MV, ddMV (Genesis), immune stimulating complex (iscom) based adjuvants and inactivated toxins.

Please replace the paragraph beginning at page 49, line 3, with the following redlined paragraph:

Following immobilization, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin, TWEEN® 20™ (Sigma Chemical Co., St. Louis, MO), heat-inactivated normal goat serum (NGS), or BLOTTO (buffered solution of nonfat dry milk which also contains a preservative, salts, and an antifoaming agent). The support is then incubated with a biological sample suspected of containing specific antibody. The sample can be applied neat, or, more often, it can be diluted, usually in a buffered solution which contains a small amount (0.1%-5.0% by weight) of protein, such as BSA, NGS, or BLOTTO. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of antibody that specifically binds WT1 within a sample containing such an antibody. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound antibody. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Please replace the paragraph beginning at page 49, line 19, with the following redlined paragraph:

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% TWEEN® 20™~~Tween 20™~~. A detection reagent that binds to the immunocomplexes and that comprises a reporter group may then be added. The detection reagent is incubated with the immunocomplex for an amount of time sufficient to detect the bound antibody. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups.

Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase and glucose oxidase) may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products. Regardless of the specific method employed, a level of bound detection reagent that is at least two fold greater than background (i.e., the level observed for a biological sample obtained from a disease-free individual) indicates the presence of a malignant disease associated with WT1 expression.

Please replace the paragraph beginning at page 53, line 15, with the following redlined paragraph:

Sera from adult patients with *de nova* AML or CML were studied for the presence of WT1 specific Ab. Recombinant proteins were adsorbed to TC microwell plates (Nunc, Roskilde, Denmark). Plates were washed with PBS/0.5% ~~TWEEN~~<sup>®</sup> ~~Tween~~ 20 and blocked with 1% BSA/PBS/0.1% ~~TWEEN~~<sup>®</sup> ~~Tween~~ 20. After washing, serum dilutions were added and incubated overnight at 4°C. Plates were washed and Donkey anti-human IgG-HRP secondary antibody was added (Jackson-Immunochem, West Grove, PA) and incubated for 2h at room temperature. Plates were washed, incubated with TMB Peroxidase substrate solution (Kirkegaard and Perry Laboratories, MA), quenched with 1N H<sub>2</sub>SO<sub>4</sub>, and immediately read (Cyto-Fluor 2350; Millipore, Bedford, MA).

Please replace the paragraph beginning at page 55, line 14, with the following redlined paragraph:

Detection of existent antibodies to WT1 in patients with leukemia strongly implied that it is possible to immunize to WT1 protein to elicit immunity to WT1. To test whether immunity to WT1 can be generated by vaccination, mice were injected with TRAMP-C, a WT1 positive tumor cell line of B6 origin. Briefly, male B6 mice were immunized with 5 x 10<sup>6</sup> TRAMP-C cells subcutaneously and boosted twice with 5 x 10<sup>6</sup> cells at three week intervals. Three weeks after the final immunization, sera were obtained and single cell suspensions of

spleens were prepared in RPMI 1640 medium (GIBCO®) with 25µM β-2-mercaptoethanol, 200 units of penicillin per ml, 10mM L-glutamine, and 10% fetal bovine serum.

Please replace the paragraph beginning at page 57, line 18, with the following redlined paragraph:

B6 mice were immunized with a group of WT1 peptides or with a control peptide. Peptides were dissolved in 1ml sterile water for injection, and B6 mice were immunized 3 times at time intervals of three weeks. Adjuvants used were CFA/IFA, GM-CSF, and ~~Mentinide~~MONTANIDE®. The presence of antibodies specific for WT1 was then determined as described in Examples 1 and 2, and proliferative T cell responses were evaluated using a standard thymidine incorporation assay, in which cells were cultured in the presence of antigen and proliferation was evaluated by measuring incorporated radioactivity (Chen et al., *Cancer Res.* 54:1065-1070, 1994). In particular, lymphocytes were cultured in 96-well plates at  $2 \times 10^5$  cells per well with  $4 \times 10^5$  irradiated (3000 rads) syngeneic spleen cells and the designated peptide.

Please replace the paragraph beginning at page 110, line 5, with the following redlined paragraph:

Peptide binding to C57Bl/6 murine MHC was confirmed using the leukemia cell line RMA-S, as described by Ljunggren et al., *Nature* 346:476-480, 1990. In brief, RMA-S cells were cultured for 7 hours at 26°C in complete medium supplemented with 1% FCS. A total of  $10^6$  RMA-S cells were added into each well of a 24-well plate and incubated either alone or with the designated peptide (25ug/ml) for 16 hours at 26°C and additional 3 hours at 37°C in complete medium. Cells were then washed three times and stained with fluorescein isothiocyanate-conjugated anti D<sup>b</sup> or anti-K<sup>b</sup> antibody (PharMingen, San Diego, CA). Labeled cells were washed twice, resuspended and fixed in 500ul of PBS with 1% paraformaldehyde and analyzed for fluorescence intensity in a flow cytometer (Becton-Dickinson ~~FACSCalibur~~FACSCALIBUR®™). The percentage of increase of D<sup>b</sup> or K<sup>b</sup> molecules on the

surface of the RMA-S cells was measured by increased mean fluorescent intensity of cells incubated with peptide compared with that of cells incubated in medium alone.

Please replace the paragraph beginning at page 115, line 10, with the following redlined paragraph:

Mononuclear cells were isolated by density gradient centrifugation, and were immediately frozen and stored at -80°C until analyzed by RT-PCR for the presence of WT1 specific mRNA. RT-PCR was generally performed as described by Fraizer et al., *Blood* 86:4704-4706, 1995. Total RNA was extracted from 10<sup>7</sup> cells according to standard procedures. RNA pellets were resuspended in 25 µL diethylpyrocarbonate treated water and used directly for reverse transcription. The zinc-finger region (exons 7 to 10) was amplified by PCR as a 330 bp mouse cDNA. Amplification was performed in a thermocycler during one or, when necessary, two sequential rounds of PCR. ~~AmpliTaq~~ AMPLITAQ® DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT), 2.5 mM MgCl<sub>2</sub> and 20 pmol of each primer in a total reaction volume of 50µl were used. Twenty µL aliquots of the PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. The gels were photographed with ~~Polaroid~~ POLAROID® film (Polaroid 667, Polaroid Ltd., Hertfordshire, England). Precautions against cross contamination were taken following the recommendations of Kwok and Higuchi, *Nature* 339:237-238, 1989. Negative controls included the cDNA- and PCR-reagent mixes with water instead of cDNA in each experiment. To avoid false negatives, the presence of intact RNA and adequate cDNA generation was evaluated for each sample by a control PCR using β-actin primers. Samples that did not amplify with these primers were excluded from analysis.